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Review

H^+ V-ATPase-Energized Transporters in Brush Border Membrane Vesicles from Whole Larvae of *Aedes aegypti*

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ABSTRACT

Brush border membrane vesicles (BBMVs) from **W**hole larvae of *Aedes aegypti* (AeBBMV**W**s) contain an H^+ V-ATPase (V), a Na^+/H^+ antiporter, NHA1 (A) and a Na^+-coupled, nutrient amino acid transporter, NAT8 (N), VAN for short. All V-ATPase subunits are present in the *Ae. aegypti* genome and in the vesicles. AgNAT8 was cloned from *Anopheles gambiae*, localized in BBMs and characterized in *Xenopus laevis* oocytes. AgNHA1 was cloned and localized in BBMs but characterization in oocytes was compromised by an endogenous cation conductance. AeBBMVWs complement *Xenopus* oocytes for characterizing membrane proteins, can be energized by voltage from the V-ATPase and are in their natural lipid environment. BBMVs from caterpillars were used in radio-labeled solute uptake experiments but ~10,000 mosquito larvae are needed to equal 10 caterpillars. By contrast, functional AeBBMVWs can be prepared from 10,000 whole larvae in 4 h. Na^+-coupled ^{3H}phenylalanine uptake mediated by AeNAT8 in AeBBMVs can be compared to the Phe-induced inward Na^+ currents mediated by AgNAT8 in oocytes. Western blots and light micrographs of samples taken during AeBBMVW isolation are labeled with antibodies against all of the VAN components. The use of AeBBMVWs to study coupling between electrogenic V-ATPases and the electrophoretic transporters is discussed.

Contents

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1. Prologue

As a graduate student, I was astounded by the way that Judy Horowitz dared to challenge the likes of E.O. Wilson, Tom Eisner, Jim Watson and Peter Medawar at Carroll M. Williams' Tea Sessions in the late 1950s. John Willis was more than astounded and gave Judy his name. Through the years, whenever a difficult problem in insect physiology arose, Judy Willis has been just a phone call away. She persuaded my group to turn to *Manduca sexta* in attempts to clone a nutrient amino acid transporter; the result was KAAT1. Geoffrey Chaucer must have been thinking of Judy six centuries ago, except he got the gender wrong and his spelling was atrocious.

"And gladly wolde she lerne, and gladly teche"

Spurred on by recollections of Judy Willis's intellectual courage, I persuaded my colleagues to help me attempt this paper, not as a review of accomplished results but as a perspective on the vast new area of membrane transport research that could be carried out on brush border membrane vesicles (BBMV) from whole dipteran larvae, if their validity could be confirmed. Meanwhile, the need for basic research that could lead to a better understanding of disease vector mosquitoes and to the development of environmentally safe mosquitocides has taken on a new urgency. The West Nile fever story has demonstrated deficiencies in our ability to deal with emerging, mosquito-born pathogens, whether they are introduced inadvertently or deliberately by bioterrorists.

2. H^+ V-ATPases are electrically coupled to NHAs and NATs in AeBBMVWs

The H^+ V-ATPase has been known for more than a quarter of a century (Cidon and Nelson, 1983; Uchida et al., 1985) but its physiological role is still not clear. The leading hypothesis is that V-ATPases transport H^+ across membranes and establish a protonmotive force (pmf) consisting of a transmembrane voltage, ΔV (or $\Delta\psi$), and a pH difference, ΔpH (Beyenbach and Wieczorek, 2006; Nelson and Harvey, 1999), by analogy with the pmf generated by the electron transport system across phosphorylating membranes (Mitchell, 1961).

$$\text{Protonmotive force (pmf in mV)} = \frac{\Delta\bar{\mu}_{H^+}}{F} = \Delta\psi - 59\,\Delta\text{pH} = \Delta V + \log\left(\frac{[H_o{}^+]}{[H_i{}^+]}\right)$$

Originally it appeared that Mitchell was referring to the pH in the bulk solutions on either side of the membrane but that was recognized as impossible in the case of alkalophilic bacteria in which the outside volume can be as great as the Pacific Ocean (Williams, 1978) and the pH can be >11 (Krulwich and Guffanti, 1986). The problem was partially resolved by Kell who proposed a system with five pH phases – an outer bulk solution, an unstirred layer adjacent to the outside of the membrane, the membrane, an unstirred layer adjacent to the inside of the membrane and the inner bulk solution (Kell, 1979, 1992), review (Harold, 1986). Harvey (2009) applied Kell's "electrodic view" to insect epithelia in an effort to explain how H^+ V-ATPases, whose sole activity is to translocate H^+ across membranes, can alkalinize the outside bulk solution to pH values that approach 11 (Fig. 1). Beyenbach had proposed such a microenvironment in the glycocalyx of the brush border in *Aedes aegypti* adults much earlier (Beyenbach, 2001). Theoretically, plasma membrane H^+ V-ATPases pose the problem in a more tractable form than phosphorylating membranes because the source of H^+ is clearly in the cell cytoplasm and an anion is clearly left behind; indeed, voltages nearly equal to those predicted from the ATP phosphorylation potential have been measured across caterpillar midgut cells (Dow and Peacock, 1989). Moreover, a mechanism by which the voltage and localized pH gradient drive secondary $K^+/2H^+$ antiport has been demonstrated in caterpillars (Azuma et al., 1995; Wieczorek et al., 1991) but, lacking a genome, it has been difficult to clone the caterpillar antiporter. Recently, a candidate antiporter (AgNHA1) has been cloned and localized in *Anopheles gambiae* (Rheault et al., 2007) and two antiporters (DmNHA1 and DmNHA2) have been cloned from *Drosophila melanogaster* and characterized in yeast ((Day et al., 2008), In addition two Na^+-coupled nutrient amino acid transporters (AgNAT6 and AgNAT8) have been cloned and localized in *An. gambiae* and characterized by electrical measurements in *Xenopus laevis* oocytes (Meleshkevitch et al., 2006, 2009). However, efforts to characterize AgNHA1 in oocytes (Harvey and Okech, 2010) were frustrated by endogenous cation conductances that are induced by the injection of RNA and other causes (Weber, 1999). Recently, Kenneth M. Sterling, Bernard A. Okech and William R. Harvey demonstrated Na^+-coupled uptake of ^{3H}-labeled phenylalanine in brush border membrane vesicles isolated from whole larvae of *Ae. aegypti* [(AeBBMVW, Fig. 2) (Okech et al., 2010)]. They showed that antibodies which label the H^+ V-ATPase (V), NHA1 (A) and NAT8 (N) [VAN for short) in gastric caeca and posterior midgut of *An. gambiae* also label the vesicles from *Ae. aegypti* (Fig. 1). AeBBMVWs have two major advantages over frog oocytes or yeast cells for characterizing newly cloned transporters. They naturally overexpress H^+ V-ATPases whose voltage-generating capacity is intact and the VAN components are all embedded in native lipids that do not distort transport function as the foreign lipids of oocytes or yeast cells may do (Hidalgo, 1987).

3. Amino acids and pH in mosquito larvae

3.1. Why amino acids?

Mosquitoes use amino acids to synthesize structural proteins while the larval mass increases ~1000-fold from eggs to 4th instar

larvae. Amino acids are also the principal energy substrates and osmolytes of mosquitoes (Clements, 1992). They are mostly taken up by Na^+: amino acid symport in the gastric caeca and posterior midgut (Boudko et al., 2005). Na^+ is scarce and would soon be depleted from the lumen except that it appears to be recycled via Na^+/H^+ antiport, resulting in a Na^+ cycle (Figs. 1 and 3). Likewise the ejection of H^+ from the cells via the H^+-V-ATPase would eventually over-alkalinize the cells and acidify the lumen. However, the postulated return of H^+ to the cells via AgNHA1 would complete an H^+ cycle. Thus both Na^+ and H^+ are thought to recycle while amino acids are absorbed (Fig. 1). This cation recycling accounts for the inability of Boudko et al. (2001) to measure fluxes of Na^+ or K^+ near midguts of mosquito larvae whereas fluxes of Cl^- were measured easily.

3.2. Why pH regulation?

How mosquito larvae regulate pH between 6.5 and 10.5 by interaction of environmentally scarce Na^+ or K^+ with metabolically produced CO_2 has fascinated scientists since Ramsay and Wigglesworth first described the phenomenon (Ramsay, 1950). A recently proposed hypothesis (Fig. 3) is that metabolic CO_2 diffuses from the cells into the ectoperitrophic space where carbonic anhydrase 9 instantaneously transforms it to HCO_3^- (Smith et al., 2007). In foregut the carbonate exists as carbonic acid and the lumen is mildly acidic. In gastric caeca H^+ exchanges for Na^+ and sodium bicarbonate sets the pH at ~8. In anterior midgut Na^+/H^+ antiport activity yields sodium carbonate which sets the pH at ~10.5. The process reverses in posterior midgut and the pH returns to near neutrality. The exchanges are driven mainly by the voltage generated by the H^+ V-ATPase (Beyenbach, 2001; Harvey, 2009; Harvey et al., 2009).

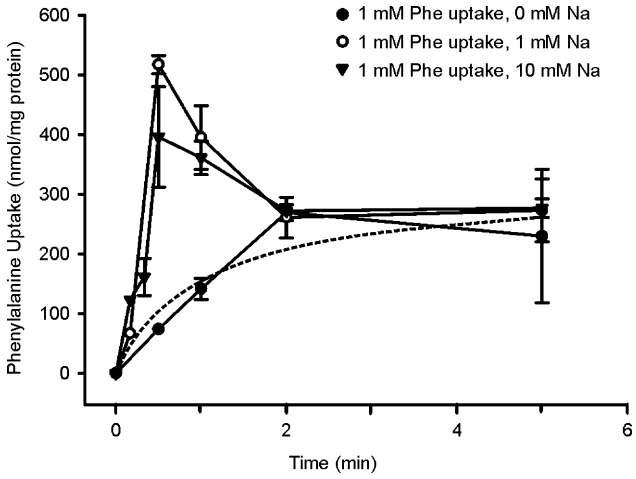

Fig. 2. Time-course of Na^+-dependent ^3H Phe uptake by BBMVW from *Ae. aegypti* 4th instar larva. Without Na^+ the time-course is typical of simple, carrier-mediated uniport (similar to calculated dashed trace). With Na^+, the time-course has an "overshoot" that is typical of Na^+: amino acid symport (K.M. Sterling, B.A. Okech and W.R. Harvey, unpublished data).

4. Log-jam in physiology of cation-coupled transporters

4.1. VAN enables study of voltage-driven transporters

Heterologous expression in *Xenopus laevis* oocytes or *Saccharomyces cerevisiae* cells is commonly used to characterize newly cloned, electrically coupled, ion transporters. Unfortunately, conductances from transcribed transporters are difficult to distinguish from endogenous cation conductances in oocytes

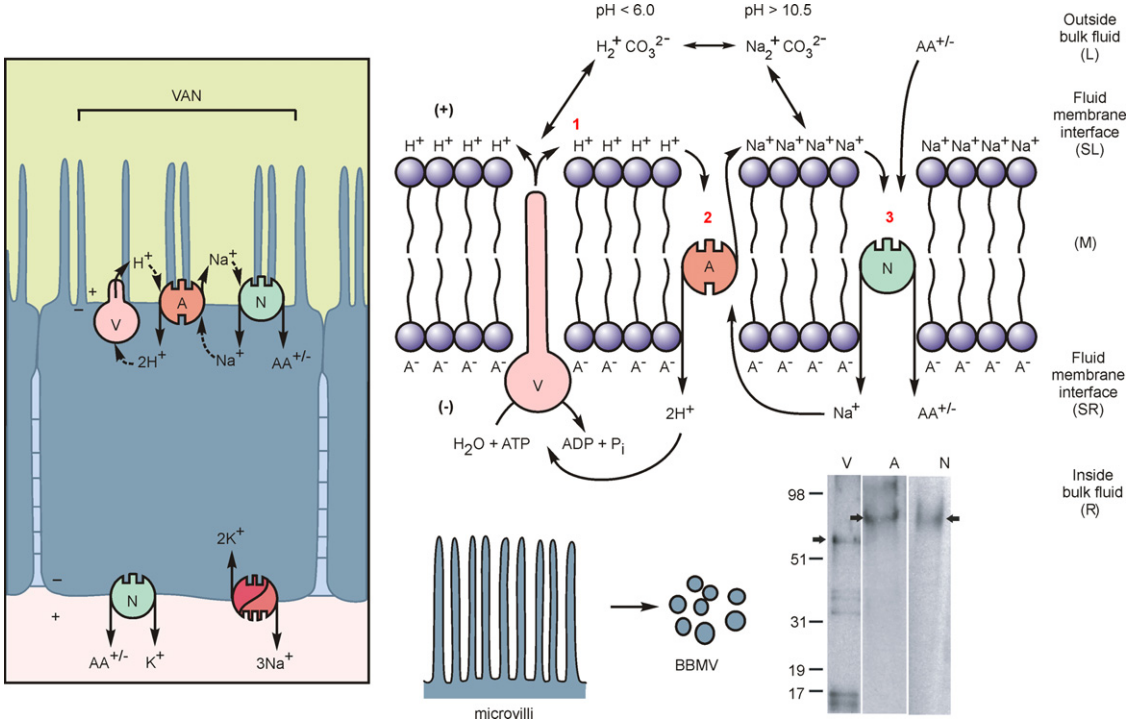

Fig. 1. Diagram of VAN in apical membrane of posterior midgut epithelial cell in *Ae. aegypti* larva. V (ATPase) drives H^+ outwardly across the lipid bilayer and generates ΔV (outside positive); ΔV drives $2H^+$ back in and Na^+ out via A (AgNHA1); ΔV also drive Na^+ stoichiometrically coupled to an amino acid into the cell via N (AgNAT8). V and N together comprise a so-called NHE_{VNAT}. In summary, the H^+ V-ATPase provides energy for an H^+ cycle linked by the voltage to a Na^+ cycle that combine to result in amino acid uptake. The VAN-containing microvilli are isolated from whole larvae as AeBBMVWs. The three VAN proteins are present in the Western blot of the vesicles. A low pH at the membrane outer surface is possible even though the external bulk pH can be >10.5 as explained by the Kell/Harvey 5-pH phase model (upper right), modified from (Harvey, 2009).

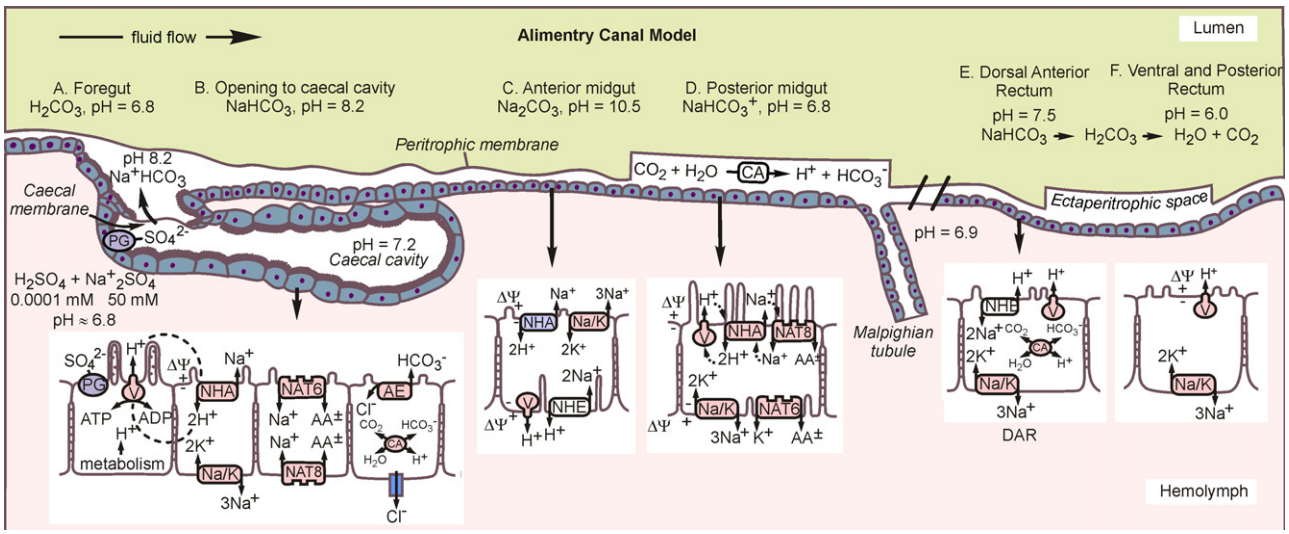

Fig. 3. Model of pH regulation and amino acid uptake in larval mosquito alimentary canal. The pH increases from foregut to anterior midgut then decreases from anterior to posterior midgut. The principal anionic component remains carbonate but the cation changes from H^+ to Na^+ to 2 Na^+ and back to H^+. The apical anion exchanger in the GC is predicted but not confirmed. The discussion focuses on VAN in posterior midgut (Harvey and Okech, 2010).

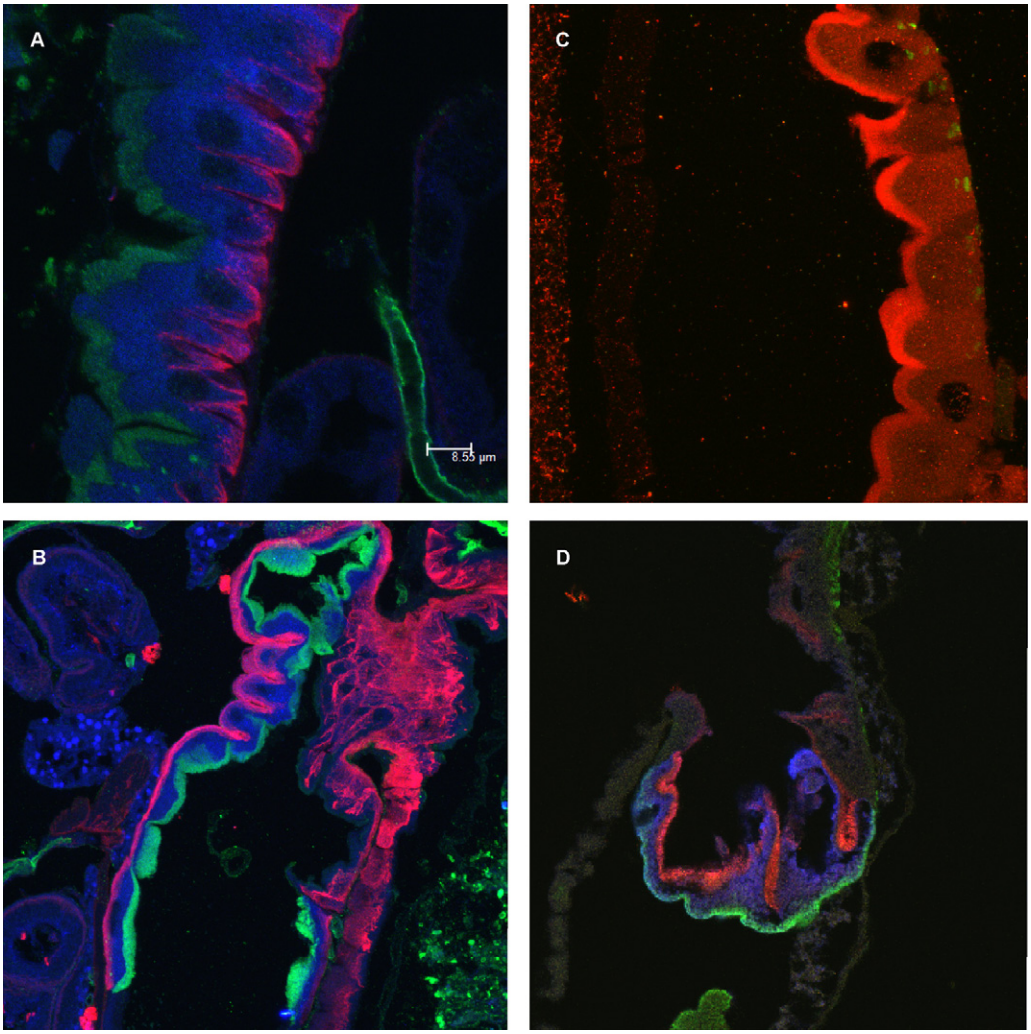

Fig. 4. Mosquito larval posterior midgut; (A and B), *An. gambiae*, apical APN2 green, basal Na/K ATPase red; (C and D), *Aedes aegypti*, apical V-ATPase red, (D) basal anion exchanger (green) (Paul J. Linser, unpublished micrographs).

and electrical measurements are virtually impossible in yeast cells. An alternative approach is provided by the strong immunolocalization of NAT8 in gastric caeca and posterior midgut (Okech et al., 2008b) and its presence in Western blots of AeBBMVWs (Fig. 1). Using epitopic sequences from the genome, antibodies were generated and used to localize transporters, both in larvae and in membrane vesicles. For example, H^+ V-ATPase (V) and two transporters, whose counterparts were cloned from *An. gambiae* – a Na^+/H^+ antiporter (AeNHA1, A) and a nutrient amino acid transporter (AeNAT8, N) – are all present in apical microvilli *in vivo* (Figs. 1, 3 and 4) and in AeBBMVW, Fig. 1); the trio is called VAN. The V-ATPase cannot be heterologously over-expressed in oocytes because of the large number of subunits (14) and the need for posttranslational processing. So its naturally high expression in AeBBMVW provides an unprecedented opportunity to study voltage coupling between the electrogenic V-ATPase and electrophoretic NHA1 and NAT8. The endogenous conductance in oocytes did not prevent electrical characterization of AgNAT8- (Meleshkevitch et al., 2006) and AgNAT6- (Meleshkevitch et al., 2009) mediated uptake in oocytes because inward currents associated with the symport could be identified and corrected for. Piermarini et al. (2009) were able to characterize the Na^+-driven Na^+/H^+ exchanger AeNHE8 in oocytes using pHi electrodes because the symport is electrically neutral and $[Na^+]$ rather than ΔV is the driving force. However, electrical studies on a Na^+/H^+ antiporter (AgNHA1) in oocytes have been difficult to interpret (Harvey and Okech, 2010). Neither were oocytes useful in attempts to characterize two NHAs cloned from *D. melanogaster*; instead DmNHA1 and DmNHA2 were characterized by molecular techniques in yeast (Day et al., 2008).

The presence of foreign lipids in the so-called 'boundary layer' adjacent to the transporter protein remains an obstacle to interpreting results when cloned mosquito transporters are over-expressed in oocytes or yeast cells. Thus, Hidalgo et al. (1976) showed that the Ca^{2+} P-ATPase of sarcoplasmic reticulum requires a minimum of ~30 boundary lipids per protein molecule and a fluid lipid environment for optimal function. If the boundary lipids are removed the ATPase activity remains but the calcium ion transport is lost (Hidalgo, 1987) (see also Thomas et al., 1982). This problem would not be present when VAN proteins are characterized in their natural lipid environment in AeBBMVW. Of course the use of AeBBMVW is limited to transporters that are naturally expressed in the apical microvilli. However, immunolabeling of gels from BBMV reveals many membrane proteins that include *Bacillus thuringiensis* subsp. *israelensis* (Bti) receptors, NATs and NHAs as well as a large number of unidentified proteins. It may even be possible to use the discarded microsomal fraction (5 Pellet, Fig. 7) to analyze transporters such as AeNHE8 which is thought to be restricted to intracellular sites (Piermarini et al., 2009).

5. Deduction that AeMVW are from brush border membranes

The hypothesis that adding $MgCl_2$ to membrane preparations will precipitate intracellular organelles such as mitochondria and endosomes but not brush border membrane vesicles has evolved over the last half century. Borgström and Dahlqvist (1958) isolated intestinal mucosal membranes by homogenization followed by differential centrifugation. Miller and Crane (1961) added filtration and low speed centrifugation steps to remove large particles and included EDTA to prevent autolysis; they introduced the term 'brush border membrane vesicles (BBMV)'. However cytoplasmic contaminants were not removed by their method. Following the key observation by Thuneberg and Rostgaard (1968) that adding Ca^{2+} or Mg^{2+} to the homogenization buffer led to electrostatic attractions with negatively charged organelles resulting in aggregate formation, Schmitz et al. (1973) added low speed differential and step gradient centrifugation to remove the aggregated microsomes, mitochondria and basolateral membrane fragments but left the BBMV in suspension. Booth and Kenny (1974) replaced Ca^{2+} by the less deliquescent Mg^{2+} and suggested that negative charges on the glycocalyx allow apical microvilli to form stable electrostatic interactions with Mg^{2+} that prevents aggregate formation. As microvilli are broken up during homogenization, the glycocalyx-protected bits of microvillus form brush border membrane vesicles that remain in the supernatant during low speed centrifugation but are spun down at forces approaching 30,000 × *g*. Hearn et al. (1981) invoked the external glycocalyx to explain why BBMVs from intestinal mucosa retain their native orientation when they are prepared by Mg^{2+} precipitation. Their electron micrographs show convincingly that microvilli vesiculate *in situ* when the mucosa is subjected to mild homogenization in sucrose-saline media (Fig. 9). The electron micrographs of larval mosquito posterior midguts (Figs. 5 and 6) suggest that this "glycocalyx-protection hypothesis" applies to mosquito midgut microvilli as well. The longitudinal section of Fig. 5B shows microfilaments running the length of the microvilli with glycocalyx on their outer surface; Fig. 6B shows a swelling at each microvillar tip. When microvilli are homogenized it appears that the microfilaments are sheared and the microvilli form vesicles with a core of microfilaments encased in cytoplasm and surrounded by plasma membrane and glycocalyx. Perhaps, as the microfilaments are sheared the non-polar plasma membrane breaks, but upon exposure to the polar external environment it reseals, resulting in spherical vesicles with the glycocalyx on the outside, like those in mucosal BBMV (Fig. 9). We predict that electron micrographs of isolated AeBBMVW will resemble both these mucosal vesicles and the cross-sections of *Culex* microvilli (Fig. 6A); in particular, the diameters of BBMVs and cross-sections of microvilli should be similar.

Perhaps, Hearn et al.'s conclusion that rat mucosal BBMV retain their natural orientation due to the glycocalyx on their outside (Fig. 9) can be extended to BBMV from mosquito midgut on the basis of their external glycocalyx (Figs. 5 and 6). If high magnification electron micrographs of isolated AeBBMVW have

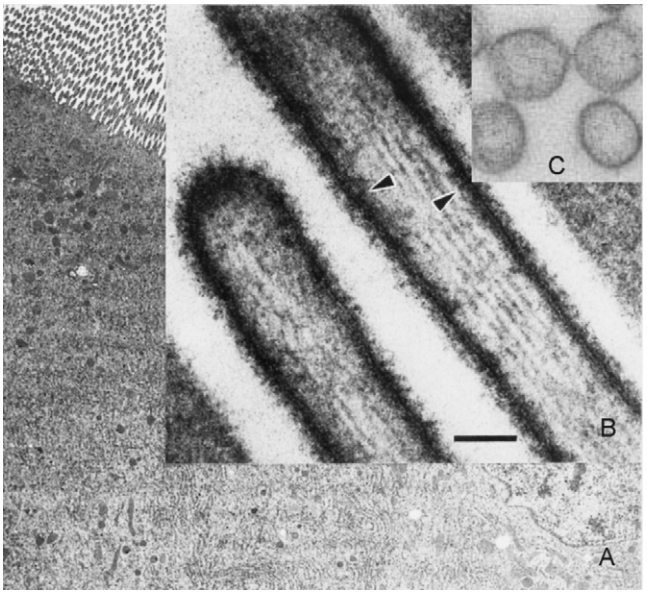

Fig. 5. (A and B) *Ae. aegypti* posterior midgut; (C) *Cx. quinquefasciatus* posterior midgut. (A) Whole cell with apical microvilli in upper left corner; (B and C) apical microvilli with external glycocalyx; (B) longitudinal section through microvilli with internal microfilaments and portasomes (arrow); (C) cross section with internal portasomes, [A and B (Zhuang et al., 1999), C (Becnel et al., 2001)].

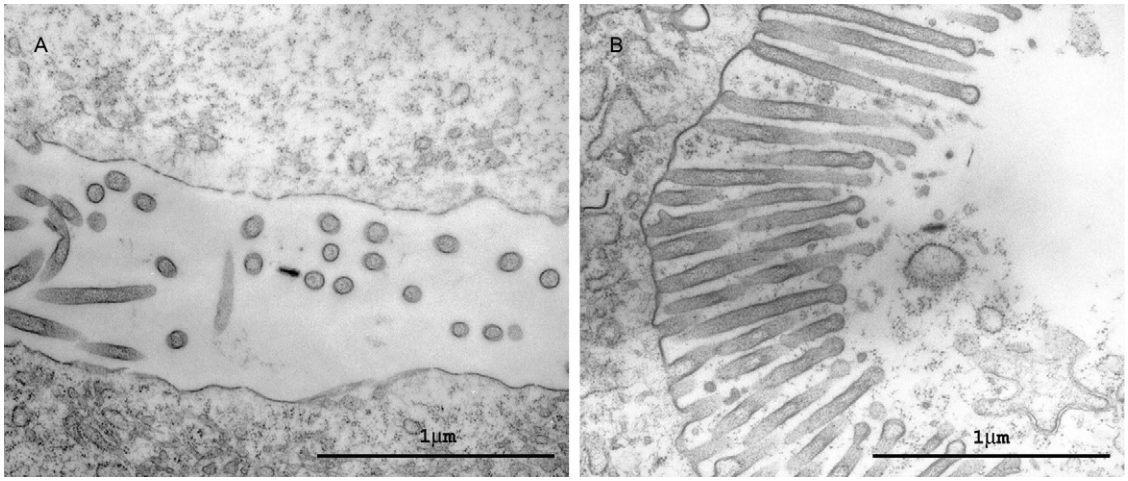

Fig. 6. *Aedes triseriatus* posterior midgut. (A) Cross-sections of apical microvilli some of which have a dense external glycocalyx. (B) Apical microvilli with bulbous distal tips, magnification 20,000× (Becnel et al., 2001).

portasomes (V_1 ATPase sectors) on the inner surface, as they do in microvilli *in situ* (Fig. 5B) the proof that the BBMV are from microvilli would be overwhelming. In turn the claim that AeBBMVW are from midgut and Malpighian tubule brush border membranes would be hard to dispute.

A highly significant advance was made when Abdul-Rauf and Ellar (1999) isolated functional BBMVs from whole *Ae. aegypti* larvae using a patented procedure that had been developed by MacIntosh (1994) on Diamondback Moth larvae. Abdul-Rauf and Ellar demonstrated that enrichment of marker enzymes in BBMV from whole larvae was similar to that in dissected midguts. Moreover, electron micrographs of the two preparations were virtually indistinguishable (Abdul-Rauf and Ellar, 1999). Fig. 7 is our scaled-up version of the Abdul-Rauf and Ellar procedure; in preliminary experiments it took Dr. Sterling ∼ four hours to produce BBMVs from 10,000 larvae in sufficient quantity to demonstrate Na^+-coupled ^{3H}Phenylalanine uptake [Fig. 2 (Okech et al., 2010)].

6. BBMV from whole larvae

6.1. VAN in AeBBMVW as ideal model for solute uptake

H^+ V-ATPases are thought to expel H^+s and generate ΔVs (outside positive) and ΔpHs (outside locally acidic) across the apical membrane of posterior midgut and gastric caeca cells [Fig. 1 (Harvey, 2009)] as well as in Malpighian tubular cells (Beyenbach, 2001). The ΔVs drive electrophoretic NATs that move Na^+ coupled to amino acids into cells and electrophoretic NHAs that move H^+ into and Na^+ out of cells. Thus, VANs are thought to cycle H^+ and Na^+ between lumen and cells while absorbing amino acids (Figs. 1 and 3). VANs in posterior midgut of mosquito larvae (Okech et al., 2008a) comprise the principal mechanism for uptake of essential nutrient amino acids that serve the ∼1000-fold growth from egg to mature larva. AeBBMVWs, unlike oocytes, naturally over-express the 14 subunit V-ATPase, so interactions between V, A and N can be studied (Figs. 1 and 10). In AeBBMVWs, V can be turned on with ATP or inhibited with bafilomycin; N can be turned on by adding amino acids or off by withholding them; A can be studied alone by omitting amino acids and ATP and imposing ΔV across the BBM with ionophores or by activating 'caged ATP' [the P^3-1-(2-nitrophenyl) ethyl ester of ATP; Calbiochem] inside cells. The V_1 sectors of the V-ATPase macromolecule [portasomes (Harvey et al., 1981)] are visible in high resolution electron micrographs of apical microvilli in many insects (Clements, 1992) including *Ae. aegypti* [Fig. 5B arrows (Zhuang et al., 1999)]; they are barely visible in the image from *Cx. quinquefasciatus* [Fig. 5C (Becnel et al., 2001)]. The dark extracellular glycocalyx (Figs. 5B and 6) allows one to tell whether BBMVs are in their natural configuration or are inside out (Hearn et al., 1981). Although BBMVs from dissected midguts of *Ae.*

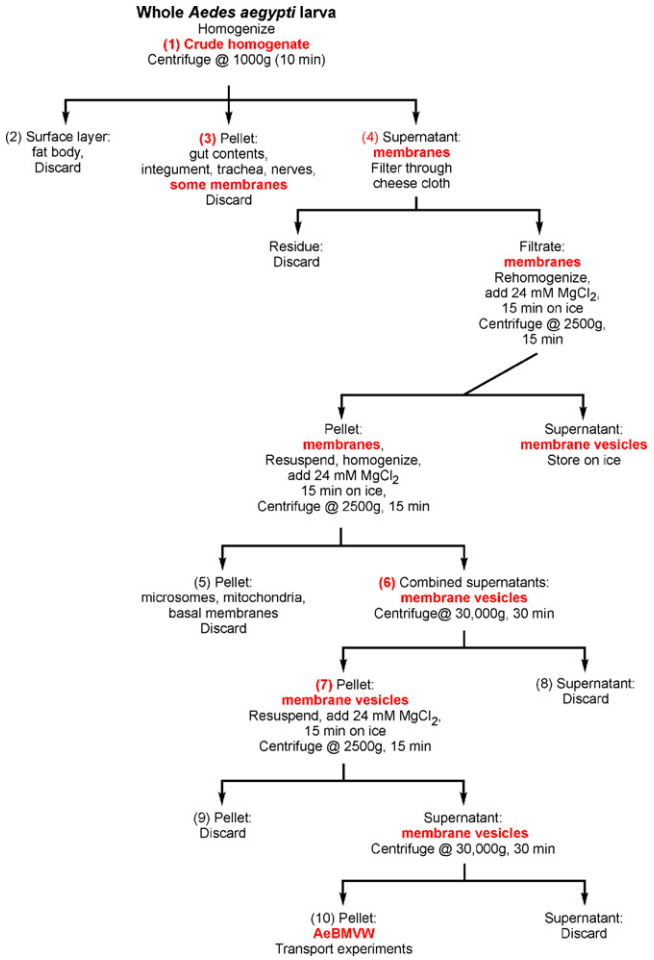

Fig. 7. Flow sheet for isolation of brush border membrane vesicles from whole *Aedes aegypti* larvae. Fractions 1, 3, 4, 6, 7 and 10 (red font) are predicted to contain increasing amounts of brush border membranes; fraction 5 is predicted to contain intracellular membranes which may include AeNHE8.

aegypti larvae would provide a supplement to *Xenopus* oocytes or yeast cells for characterizing many new transporters, thousands of dissected midguts would be needed to provide sufficient vesicles for transport studies. Isolation of the BBMV from whole larvae (Abdul-Rauf and Ellar, 1999) would solve the problem.

6.2. Larval rearing conditions are critical

The temperature and duration of larval rearing of Abdul-Rauf and Ellar (1999) must be followed carefully to obtain vesicles that retain transport capability. As larvae develop, solute transport is lost prior to pre-pupal formation when the transporting epithelium is exposed to proteolytic enzymes at the onset of midgut disintegration (Clements, 1992).

6.3. AeBBMVWs are from gastric caeca, posterior midgut and Malpighian tubules

Specific labeling of apical BBMs with antibodies against the Bti receptor, aminopeptidase 2 (APN2) and V-ATPase is clear (Fig. 4). Panels A and B show labeling for APN2 (green) versus Na/K-ATPase (a basal membrane marker protein, red) in the posterior midgut epithelium (A) and the gastric caeca (B). Panels C and D show labeling for V-ATPase in the apical brush border membrane of posterior midgut (C, red) and of the gastric caeca (D, red) confirming the earlier localization by Zhuang et al. (1999); the orientation was verified by the basal membrane marker, anion exchanger (D, green). These brush border membrane markers and others can be used to confirm that AeBBMVWs are from apical microvilli by immuno-analysis of samples from all stages of AeBBMVW preparation (samples are numbered in parenthesis in Fig. 7); the markers can be used to stain Western blots using antibodies to the Bti receptors APN1, APN2 and alkaline phosphatase (AP) as well as to V-ATPase subunit A, AgNHA1, AgNAT6 and AgNAT8, all of which stain the brush border region of posterior midgut and gastric caeca of *An. gambiae* (Okech et al., 2008a). Controls that failed to stain Western blots during AeBBMVW preparation include antibodies to Na/K ATPase and cytochrome b. Some of the BBMVs from whole *Ae. aegypti* larvae are likely to be derived from Malpighian tubules, in which the glycocalyx which surrounds the microvilli that comprise the brush border has been postulated by Beyenbach to be a region with substantially lower pH than the bulk fluid so that the proton chemical potential could power secondary transporters (Beyenbach, 2001).

If MVW are from the brush border then samples should show progressive enrichment during vesicle isolation. The density of bands in Western blots should increase during enrichment (Fractions 1, 3, 4, 6 and 7 in Fig. 7) and should decrease or be absent in fractions from which vesicles are removed (Fractions 2, 5, 8 and 9 in Fig. 7). The total enrichment in membrane vesicles isolated from whole larvae should be at least as great as in vesicles from dissected midguts. The progressive enrichment should also appear in confocal and EM images of the fractions. The surface layer (Fig. 7, Fraction 2) from the 1000 *g* centrifugation of the initial homogenate should contain fat droplets and the pellet should show fragments of integument, tracheae and nerves as well as some membranes. After filtering through cheese cloth, large debris should be absent from the filtrate. Membrane vesicles from the final pellet after the 30,000 g spin should look the same in electron micrographs from whole larva as from dissected midgut, as they do in the micrographs of Abdul-Rauf and Ellar (1999). Portasomes [V_1-ATPase particles (Gruber et al., 2000)] should be visible on the inside surface and glycocalyx on the outside surface of the vesicles, just as they are visible in electron micrographs from sections of larvae (Fig. 5). The time-course of amino acid uptake by the vesicles should show an initial "overshoot" due to the additional driving force from the imposed Na^+ gradient followed by a gradual decline to the equilibrium level as Na^+ enters the cells and reverses the Na^+ gradient (Fig. 2). The presence of all of these features would be good evidence that the membrane vesicles from whole mosquito larvae are AeBBMVs. The use of tissue organization and ultrastructure for isolating membrane vesicles follows the strategy used to isolate brush border membranes from many sources (Kinne, 1976; Murer et al., 1976). The use of AeBBMVW for transport studies is an expansion of their use by Abdul-Rauf and Ellar (1999) for studies on Bti receptors.

6.4. Problems to resolve

The belief that functional BBMV cannot be isolated from whole mosquito larvae is a major roadblock to mosquito membrane biochemistry. Adequate funding for midgut transport research using membrane vesicles from whole mosquito larvae has been rejected repeatedly by federal agencies with comments such as: **"***There is significant concern on the source of BBMV that have been used and are being proposed. These will be prepared from whole larvae, and with due respect to Abdul-Rauf Ellar (1999), these BBMV bear limited resemblance to BBMV prepared from the alimentary canal itself, even though both are vesicles. It is clear transport will be observed, but then indicating that it represents midgut transport is a stretch. While it may be tedious, it is essential the guts are dissected and BBMV prepared from these dissected tissues used".*

An important long-term goal is to characterize the first NHA1, a unique transporter that is present in all eukaryotic phyla. For this

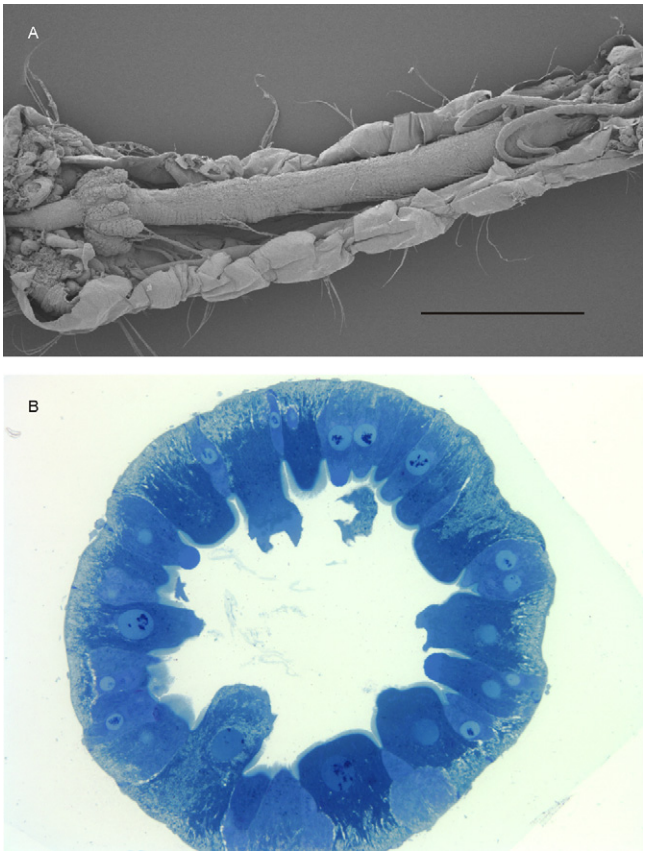

Fig. 8. *Ae. aegypti* whole 4th instar larva. (A) Scanning electron micrograph of larvae opened by incision along the dorsal side of the exoskeleton; bar ~20 mm. Note that the midgut is suspended from the integument only by tracheal trunks and nerve fibers. (B) Posterior midgut cross section with peritrophic membrane removed; the apical brush border lines the lumen. The one-cell thick epithelium has cells that range in size from basal regenerative cells to tall columnar cells, [A. (Linser et al., 2007), B. (Zhuang et al., 1999)].

purpose it only matters that the membrane vesicles from whole larvae contain NHA1 along with the H^+ V-ATPase and NAT8, so the criticism is irrelevant. However, there are several reasons to believe that the vesicles isolated from whole larvae are indeed derived from the brush border. The midgut, which has a one-cell-thick, unfolded epithelium, occupies ~1/3rd of a mosquito larva and is suspended by trachea and nerves from the integument so the two are easily separated [Fig. 8A from Linser et al., 2007]. The brush border is prominent along the luminal (apical) surface of the cells [Fig. 8B from Linser et al., 2007]. Both the brush border and the vesicles stain with brush border markers including antibodies to three Bti receptors as well as to AgNAT8, AgNHA1 and V-ATPase Subunit A (see Western blot in Fig. 1). Non-brush border markers include antibodies to Na/K ATPase (Fig. 4) cytochrome oxidase and lysosomes (data not shown). Especially convincing evidence that the vesicles are functional BBMVs is the Na^+-coupled amino acid uptake by vesicles that have been isolated from whole *Ae. aegypti* larvae (Fig. 2).

A second problem is that the antibodies used to label AeNHA1 and AeNAT8 were raised against epitopes that were selected from the *An. gambiae* rather than the *Ae. aegypti* genome. Thus, NHA1 and NAT8 were cloned and localized from *An. gambiae* larvae but the Abdul-Rauf/Ellar protocols are for *Ae. aegypti*. Another reason for working with *Ae. aegypti* is that there are containment issues when working with the dangerous, *Plasmodium falciparum* vector, *An. gambiae*. The same epitopic sequences that were selected to prepare antibodies to AgNAT8 might be chosen to prepare antibodies to AeNAT8 since the sequences are highly identical:

```
NAT8 Epitope

Ag 619  GPIDPATHYEYKKFIDED

        GPID   + EYKKFI+ED

Ae 615  GPIDANPNQEYKKFINED
```

However, epitopes chosen for AgNHA1 are not so likely to be chosen for AeNHA1.

```
NHA1 Epitope 1

Ag 94   EALEKIERDYD----NSRL

        E L KI ++Y+    N+ +

Ae 66   EVLAKINQEYETGQQNAHI

NHA1 Epitope 2

Ag 535  LKTVMSNENRTEEEVHY

        LK V S ++R+EEE HY

Ae 511  LKAVAS-QHRSEEEKHY
```

This issue could be resolved easily by raising antibodies against *Ae. aegypti* NAT8 and NHA1 and using them to compare BBMV isolated from whole larvae (Abdul-Rauf and Ellar, 1999) with those isolated from dissected midguts (Wolfersberger et al., 1987).

Yet another reason for selecting *Ae. aegypti* is that long microvilli are present on the apical membrane in gastric caeca, posterior midgut and Malpighian tubules but not in anterior midgut of this mosquito (Zhuang et al., 1999). Since there are but stubs of microvilli in anterior midgut, there can be no enclosed mitochondria and no worry about mitochondrial contamination. By contrast long microvilli enclose mitochondria in anterior midgut of *An. gambiae* (B. Okech, unpublished observations); so, despite the likelihood that mitochondrial fragments would be precipitated by $MgCl_2$, AgBBMVW might be contaminated by mitochondrial ATP synthases and antiporters.

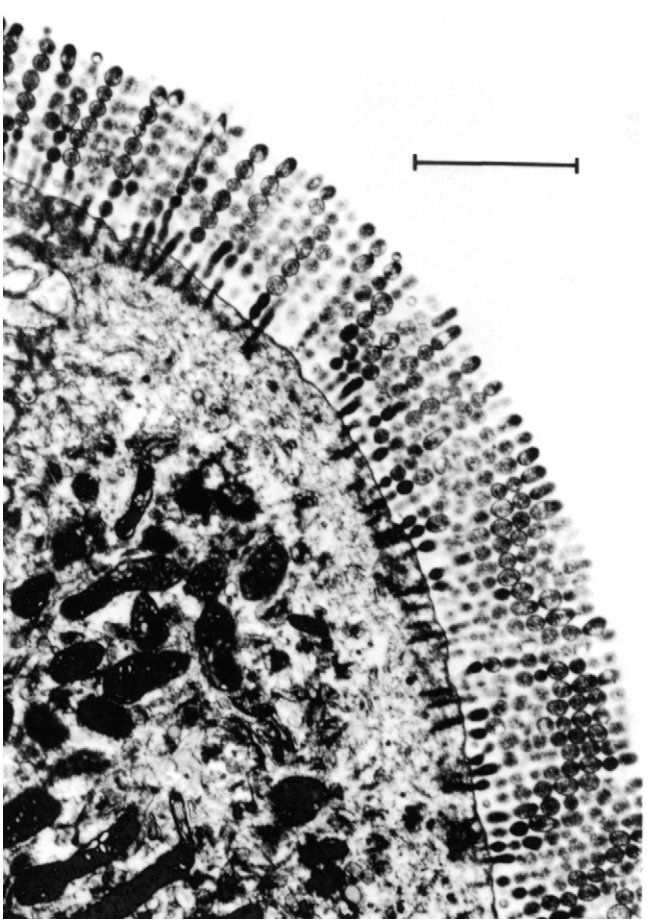

Fig. 9. Electron micrograph of brush border from rat duodenal mucosa after mild homogenization in a buffer containing 250 mm sucrose, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 5 mM Tris/HCl at pH 7.4, showing that vesiculation of microvilli occurs *in situ* and ensures that the vesicles will be 'right-side-out" (Hearn et al., 1981).

Despite skeptical criticisms, the only difference between isolating BBMV from whole larvae vs. dissected midguts is that homogenates of whole larvae are strained through cheese cloth to remove the integument and other debris before the first $MgCl_2$ precipitation step (Fig. 7) whereas homogenates of dissected midguts are processed directly. Although AeBBMVW have not been used previously for transport studies they have been used to study Bti receptors. Thus, Zhang et al. (2008) used isolated MV from frozen whole *An. gambiae* larvae (furnished by the Malaria Research and Reference Reagent Resource Center) to show that the 106-kDa aminopeptidase is a specific binding protein and a putative BtCry11Ba receptor. Silva-Filha et al. (1997) used "brush border membrane fractions" from whole larvae to show direct binding by ^{3H}*Bacillus sphaericus* endospores.

7. Comparison of ^{3H}Phe uptake in AeBBMVWs and *Xenopus* oocytes

7.1. Rationale

To validate the use of MVWs in transport experiments, the characteristics of AeNAT8 in ^{3H}Phe uptake experiments on AeBBMVW can be compared with those from 2-electrode voltage clamp measurements on AgNAT8 in frog oocytes (Meleshkevitch et al., 2006). Thus AgNAT8 prefers phenylalanine or tyrosine over tryptophan and barely transports any other amino acid; the

endogenous conductance in oocytes affects all values equally and can be subtracted out. Radio-labeled amino acid uptake by BBMVW in the presence or absence of Na^+ can be determined using the rapid filtration assay (Ahearn et al., 1985; Sterling et al., 2009). Other characteristics that could be studied in vesicles include: osmolarity of uptake solutions to assess leakiness, cation and anion preferences and pH of external medium. Especially useful would be the effects of inhibitors such as bafilomycin (V-ATPase), amiloride and 5-ethylisopropyl amiloride (NHEs) and the effects of prospective inhibitors of AeNHA1 and AeNAT8. For the later the effects of representative amino acids Arg, Glu, Tyr, Trp, Leu or Met, in the outside buffer on the uptake of ^{3}H-Phe can be measured at optimal NaCl concentrations to determine the amino acid preference spectrum of AeNAT8. Amino acid derivatives can be screened as putative inhibitors using methods developed by Hennigan et al. for caterpillar BBMV (Hennigan et al., 1993a,b). Specific uptake activity (CPM/nmol) can be determined by methods initiated by Hevesy [see Ussing and Zerahn, 1951].

8. Characterization of AeNHA1 in AeBBMVW

Of particular interest is the effect of voltage upon the on Na^+/H^+ exchange by NHA1 to determine if they function like bacterial NHA1s, which are voltage driven. Since the isolated BBMVs are likely to be right-side-out (Hearn et al., 1981), the V_1 H^+ V-ATPase sectors (portasomes) will be on the inside (Fig. 10). A few minutes after isolation the inside ATP will be depleted but "caged ATP" that is added during vesicle isolation will persist in safe light. A flash of activating light will release the ATP, which will activate the H^+ V-ATPase and impose a large voltage (inside negative) for ~60 s (Thomas et al., 1982). The exchange of Na^+ for H^+ in AeBBMVWs expressing AeNHA1 can be measured by the efflux of $^{22}Na^+$ in terms of: (1) ΔV vs. $\Delta[Na^+]$ as driving force, (2) direction of transport, (3) cation selectivity, (4) cation stoichiometry, (5) cation uptake and inhibitor kinetics using the methods developed in studies on AeNAT8. The procedures for working with caged ATP are modeled after those used by Thomas and associates (Lewis and Thomas, 1992; Thomas et al., 1982) for studying Ca^{2+} ATPase in sarcoplasmic vesicles.

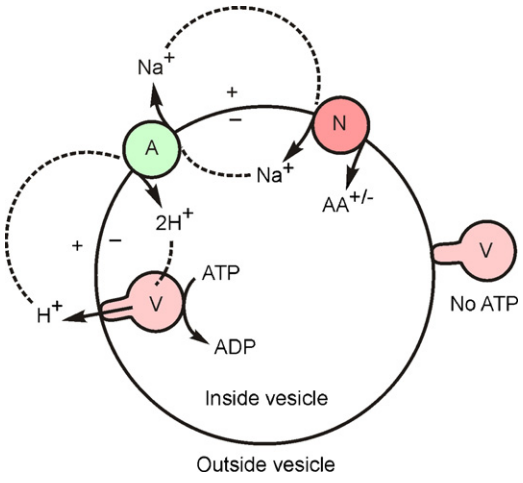

Fig. 10. Cartoon of an AeBBMVW showing amino acid uptake mediated by H^+ V-ATPase (V), AgNHA1 (A) and AgNAT8 (N) comprising VAN. When ATP is hydrolyzed H^+ is extruded by V but remains in the unstirred layer adjacent to the membrane; $2H^+$ are driven back in and 1 Na^+ is driven out by A while the Na^+ and an amino acid are driven in by the voltage. The net result is that H^+ cycles via V and A, Na^+ cycles via A and N and amino acids are taken up by N (Harvey, 2009). Moreover, H^+ extrusion by V together with Na^+ uptake by N is equivalent to the action of a Na^+/H^+ exchanger and has been called NHEVNAT(Harvey et al., 2009).

8.1. Broader implication of work on NHA1

Convincing confirmation of the hypothesis that AeBBMVW are valid objects for transport studies (whether from brush borders or not) would open a floodgate of major research projects. As noted above AgNHA1 and AeNHA1 are important as models since NHAs are present in every genome (Brett et al., 2005). Their localization in mosquito midgut cells and the lack of sufficient ionic driving forces there suggests that they may be driven by the voltage generated by the H^+ V-ATPase which, by analogy to caterpillar $K^+/2H^+$ antiporter (Azuma et al., 1995; Wieczorek et al., 1991), is postulated to translocate $2H^+$ inwardly coupled to a Na^+ outwardly (Fig. 1). This Na^+/H^+ antiport is in the opposite direction of classical NHE exchange and would provide a new mechanism for pH and salt regulation. On the practical side, AeBBMVWs can be obtained in massive quantities and could be used in high-throughput screens for inhibitors of NHAs to develop as environmentally safe mosquitocides.

9. Proton paradigm in animal membranes

9.1. Energization of secondary transporters by H^+ V-ATPases

As discussed above, the presence of H^+ V-ATPase in AeBBMVWs allows one to impose a voltage and study electrophoretic carriers. The determination of voltage effects, ion selectivity, substrate spectra, inhibitor kinetics and other characteristics of endogenous NHA1 in AeBBMVWs can provide information that has evaded 'oocyte studies'. For example, its location in the mosquito midgut and its phylogeny suggest that AeNHA1 is voltage driven and amiloride insensitive (Rheault et al., 2007). Its relationship to the V-ATPase and NAT8 suggests that AeNHA1 mediates inward H^+ transport coupled to outward Na^+ transport [Figs. 1, 3 and 10 (Okech et al., 2008a; Rheault et al., 2007)]. In this respect, mosquito NHAs resemble alkalophilic bacterial NHAs (Krulwich et al., 1998; Padan et al., 2005). It is not unlikely that all metazoan NHAs share these transport properties. If so, a new paradigm for membrane energization in eukaryotic epithelia would emerge – pH regulation that is mediated by classical, $[Na^+]$- driven, NHEs would be augmented by pH regulation that is mediated by novel, voltage driven, NHAs (Harvey, 2009). In a broader sense AeBBMVWs would complement *Xenopus* oocytes as tools for characterizing certain newly identified transporters.

9.2. Alkalophilic bacteria, mammalian small intestine and mosquito midgut

The H^+ V-ATPase-generated, H^+ electrochemical gradient model is presented here as a complement to the classical Na^+/K^+ P-ATPase-generated, Na^+ gradient model for explaining how nutrients and ions are transported across animal cell membranes. As noted above the H^+ electrochemical gradient, $\Delta\bar{\mu}_{H^+}/F$, consists of an H^+ concentration component, $59mv \cdot \log([H^+]_O/[H_i^+])$, and a voltage component, ΔV ($\Delta\psi$). Introduced by Peter Mitchell in (1961), $\Delta\bar{\mu}_{H^+}$ is the driving force for secondary H^+-coupled solute transport in bacteria, yeast, plants, many insect epithelia, frog skin and many mammalian epithelial cells, e.g. in kidney collecting ducts, bone osteoclasts and ocular ciliary epithelia (Wieczorek et al., 1999). Increasing awareness of H^+-coupling in amino acid and other secondary transporters in mammalian small intestine (Thwaites and Anderson, 2007) has provided a quantum boost in scientific significance and health relevance to studies of H^+ coupled (Na^+ or K^+)/H^+ antiporters (NHAs) in the *An. gambiae* and *Ae. aegypti* larval midguts. Their value is illustrated by comparing three systems in which H^+s are driven into cells by $\Delta\bar{\mu}_{H^+}$ (Fig. 11). In all three cases the bulk extracellular phase is alkaline and an H^+-rich

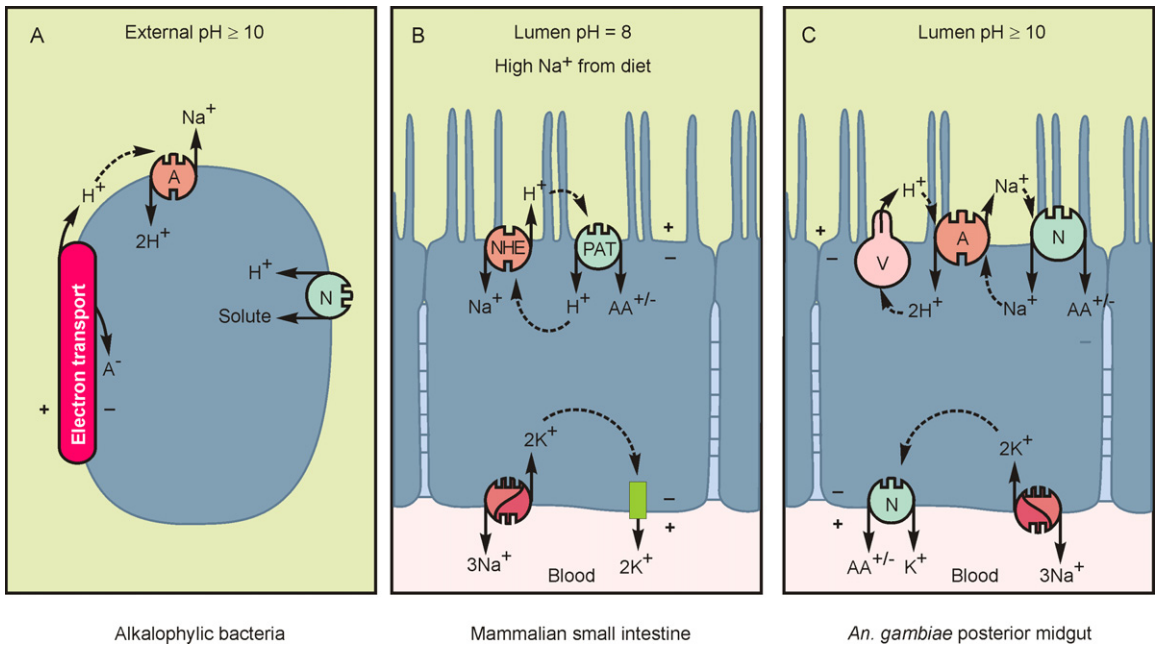

Fig. 11. Cartoons that illustrate the generation of proton electrochemical differences, consisting of outside positive voltage differences and outside low pH differences, across three classes of cell membrane. In all three cases the voltage and high H^+ concentrations drive secondary H^+-coupled transport. In *An. gambiae* posterior midgut $Na^+/2H^+$ antiport provides Na^+ for voltage-drive Na^+: amino acid uptake; H^+ and Na^+ both cycle between cell and lumen while amino acids are taken up into the cells.

microenvironment adjacent to the cell membrane is postulated to explain how H^+s are driven into the cells.

In alkalophilic bacteria (Fig. 11A) the electron transport system ejects H^+ toward the external environment (which can be as large as the Pacific Ocean), but it is held in a microenvironment adjacent to the membrane (Kell, 1979) thus directly generating both $\Delta[H^+]$ and $\Delta\psi$. The $\Delta\bar{\mu}_{H^+}$ drives Na^+ out and H^+ into the cells by a Na^+/H^+ antiporter. It also drives several well-characterized H^+-coupled secondary transporters (Krulwich and Guffanti, 1992).

In mammalian small intestine (Fig. 11B) ATP hydrolysis, via the Na^+/K^+ P-ATPase, expels Na^+ from the base of the cells and, with high dietary Na^+ in the lumen, generates an inward $[Na^+]$ gradient across the apical membrane. The $[Na^+]$ gradient drives a secondary Na^+/H^+ exchanger (NHE3) which generates a trans-apical $[H^+]$ gradient. Meanwhile, the influx of K^+ via the Na^+/K^+ ATPase generates an outward $[K^+]$ gradient which, via a K^+ channel, generates a basal K^+ diffusion potential, ΔV, which appears across the apical membrane as well. The apical $[H^+]$ gradient and ΔV form an apical $\Delta\bar{\mu}_{H^+}$ that drives tertiary, electrophoretic, H^+-coupled amino acid cotransport (via PAT1) or di/tripeptide cotransport PepT1(Thwaites and Anderson, 2007).

In *An. gambiae* posterior midgut (Fig. 11C) ATP hydrolysis via the H^+ V-ATPase (V) drives H^+ across the apical plasma membrane, leaving behind its anion (A^-) which transiently holds the H^+ in a microenvironment at the membrane surface and creates a membrane potential, thereby directly generating a $\Delta\bar{\mu}_{H^+}$ across the apical membrane. The $\Delta\bar{\mu}_{H^+}$ drives electrophoretic $Na^+/2H^+$ antiport (A) which replaces $\Delta\bar{\mu}_{H^+}$ by $\Delta\bar{\mu}_{Na^+}$ that in turn drives Na^+-coupled amino acid symport (co-transport) (N) [Fig. 1 (Harvey, 2009)].

Until the early 1990s the Na^+ gradient hypothesis was the accepted explanation of how sugars, amino acids and other nutrients are taken up and how ion concentrations are regulated in animal epithelia. Thwaites and Anderson (2007) reviewed work during the past 20 years that established a "microclimate" of high $[H^+]$ at the luminal (apical) border of enterocytes as an alternative explanation (Fig. 11B). A simpler way to generates an H^+-rich microenvironment is described in Fig. 11C in which the caterpillar midgut H^+ V-ATPase drives secondary $K^+/2H^+$ antiport across the apical membrane (Wieczorek et al., 1991). The resulting K^+ microenvironment along with bulk K^+ in the lumen drive tertiary amino acid symport (co-transport). Following the suggestion by Beyenbach (2001), our work on larval mosquito transport extends this hypothesis to tertiary Na^+-coupled solute transport, in particular to amino acid cotransport [N in Figs. 1, 3, 10 and 11 (Harvey, 2009; Harvey and Okech, 2010)]. The direct H^+ V-ATPase mechanism of Fig. 11C is obviously far simpler than the indirect Na^+/K^+ mechanism of Fig. 11B.

Thwaites and Anderson (2007) argued that "...Na^+ coupling became generally accepted as the primary means of solute movement in mammalian tissues, whereas H^+ coupling was considered to be specific for plants, yeast and bacteria. Unfortunately, once such a doctrine becomes enshrined within the literature it is often difficult to dislodge from the scientific psyche." Ironically, insect physiologists have ignored the literature on H^+ -coupling in mammalian small intestine while mammalian physiologists have ignored the literature on H^+ V-ATPases in insect midguts and Malpighian tubules. Perhaps because H^+ V-ATPases were first isolated from vacuolar membranes (Cidon and Nelson, 1983; Uchida et al., 1988) (hence their name) the doctrine that they are only important in a few specialized plasma membranes is similarly enshrined within the scientific psyche. However, all eukaryotic cells contain H^+ V-ATPases within their endomembranes which are well known to recycle rapidly with plasma membranes. Thus H^+ V-ATPases are present, transiently, in all animal cell plasma membranes (Harvey and Wieczorek, 1997). Under appropriate selective pressure they may remain there and energize secondary transport in the mammalian small intestine as they do in the mosquito midgut (Fig. 11C).

It is crucially important to know if the many tertiary H^+-coupled amino acid and other solute transporters expressed in mammalian small intestine are also expressed in larval mosquito midgut. The excerpt from a phylogenetic tree (Fig. 12) shows that the four PATs in the *Homo sapiens* genome all have near neighbors in the *An. gambiae* genome. Seven other mammalian H^+-coupled transporters have *An. gambiae* near- neighbors. Likewise it is important to

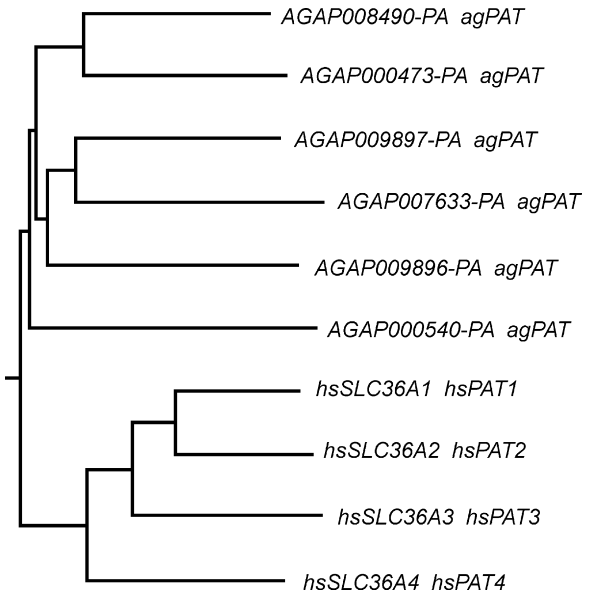

Fig. 12. Portion of phylogram showing proximity of hsPATs to agPATs, courtesy of David A. Price.

know if the primary, $\Delta\bar{\mu}_{H^+}$ -generating H^+ V-ATPase is present in the apical membrane of mammalian small intestine, where H^+-coupled secondary transporters are active, not only in nutrient and ion transport but also in the transport of drugs used to treat bacterial infections, hypertension, epilepsy, cancer and other diseases (Thwaites and Anderson, 2007).

10. Fields of dreams

10.1. Mitchell revisited – 5-pH phase model

VAN in membrane vesicles can serve as a model for the proton paradigm in which voltage and $\Delta[H^+]$ rather than $\Delta[Na^+]$ provide energy for solute transport. In particular VAN in AeBBMVW provides a means to test the hypothesis that Mitchell's protonmotive force should be reinterpreted in terms of a transmembrane voltage and five rather than three pH phases [Fig. 1 (Harvey, 2009)] as discussed above. Procedures used on phosphorylating membranes of alkalophilic bacteria, chloroplasts and mitochondria [reviews (Cherepanov et al., 2003; Mulkidjanian and Cherepanov, 2006)] can be used on AeBBMVW. Briefly, a pH responsive fluorescent label could be attached to the membrane, e.g. via a Bti receptor such as APN2 and a second pH responsive label could be attached to a soluble protein in the bulk phase. Vesicles can be prepared with caged ATP inside (Fig. 10). A flash of light with the proper wave-length will release the ATP which in turn will activate the H^+ V-ATPase and translocate H^+ to the membrane external surface (Fig. 1). The 5 pH-phase model predicts that the membrane-bound label will signal a local decrease in pH within microseconds whereas the label in the bulk solution will respond only after milliseconds. Again, the protocols can be based on those developed by D.D. Thomas and associates (Thomas et al., 1982).

10.2. Role of AeNHE8 in mosquito cation exchange

The Gill and Beyenbach laboratories disagree regarding the localization and role of the Na^+/H^+ exchanger, AeNHE8. Based upon immuno-localization studies, Kang'ethe et al. (2007) concluded that AeNHE8 is localized in the apical plasma membrane of Malpighian tubules, gastric caeca and rectum where they speculate that "AeNHE8's activity is coupled to the proton gradient created by the apical V-ATPase to secondarily drive Na- or K-extrusion across the tubule's epithelia". The Kang'ethe et al. view is challenged by Piermarini et al. (2009) who failed to confirm plasma membrane localization and presented evidence that AeNHE8 is an electroneutral intracellular cation/H^+ exchanger. How can the highly respected Gill and Beyenbach laboratories come to such opposite conclusions? One possibility is that in feeding larvae NHE_{VNAT}s (discussed below) may replace classical NHEs which are temporarily targeted to an intracellular location. However, during molts and in prepupae Na^+-coupled amino acid uptake ceases, NHE_{VNAT}s would stop functioning, and it is then that the classical NHEs would be targeted to the plasma membrane, where they would expel metabolic acids. To settle the point would require very careful monitoring of larvae to avoid studying what appear to be large 4th instar larvae but are actually prepupae in which transport is shut down as the degradation of the midgut is initiated (Clements, 1992). The flow chart for preparing AeBBMVW (Fig. 7) suggests a more direct way to decide which view is correct. If AeNHE8 is in endomembranes (as reported by Piermarini et al.) it would be precipitated by $MgCl_2$ and an antibody to it would label the discarded microsomal fraction (Sample 5 Pellet). On the other hand if AeNHE8 is in the apical membrane (as reported by Kang'ethe et al.) then the antibody would label the AeBBMVW fractions (Samples 1, 4, 6, 7, and 10).

Both of these studies on *Ae. aegypti* along with studies on *Anopheles* (Rheault et al., 2007) and *Drosophila* (Day et al., 2008) suggest that NHAs are the best candidates for apical cation/H exchangers in Malpighian tubules, gastric caeca and posterior midgut. Piermarini et al. noted that that the potential of AgNHA1 for $K^+/2H^+$ or $Na^+/2H^+$ exchange (Rheault et al., 2007) would allow the antiport to be driven by the large voltage across the apical membrane of principal cells that is generated by the H^+ V-ATPase. The presence of the V-ATPase and NHA1 in AeBBMVW provides a simple system in which to evaluate the hypothesis that AeNHA1 is the mosquito equivalent of the caterpillar $K^+/2H^+$ antiporter (Azuma et al., 1995; Wieczorek et al., 1991).

10.3. Do $AeNHE_{VNAT}$s relieve selective pressure for expression of classical NHEs?

Kang'ethe et al. (2007) noted that there are only five NHEs (isoforms 3, 6, 8, 9 and 10) in dipteran genomes compared to nine in mammals and *C. elegans*. Two of them (9 and 10) are now acknowledged to be NHAs and have been renamed NHA2 and NHA1, respectively (Brett et al., 2005; Rheault et al., 2007), leaving just three classical NHEs that could carry out their traditional function of expelling metabolic acids by ejecting H^+ in exchange for Na^+ (Grinstein and Wieczorek, 1994; Orlowski and Grinstein, 1997). Harvey (2009) pointed out that H^+ V-ATPase ejection of H^+ toward the lumen while NATs are taking up Na^+ (along with amino acids) is the functional equivalent of an NHE. Since there are seven NATs in both *An. gambiae* and *Ae. aegypti* genomes, there are potentially seven so-called NHE_{VNAT}s that could relieve the selective pressure to express NHEs in dipteran larvae. Two of them, AgNAT6 (Meleshkevitch et al., 2009) and AgNAT8 (Meleshkevitch et al., 2009) have already been localized to the apical membrane of posterior midgut cells in *An. gambiae* larvae.

10.4. Tons of transporters from AeBBMVWs

During the U.S. Army's post-WWII biological warfare initiative, billions of *Ae. aegypti* adults were mass produced with plans to infect them with the yellow fever virus and use them as weapons (Lockwood, 2009; Mangold and Goldberg, 2000). This grim note from the past has a bright side; the Army demonstrated that *Ae. aegypti* larvae can be produced in whatever numbers are desired. It

may turn out that, rather than giant *Manduca sexta* caterpillars or transgenic *Escherichia coli* cells, *Ae. aegypti* larvae could be the source of crystals in which to determine the structure of H^+ V-ATPase, AeNHA1 and AeNATs 6 & 8 along with other transport proteins. Thus far the crystal structures of only V-ATPase subunit C (Drory et al., 2004) and subunit H (Sagermann et al., 2001) have been published and the structures of subunits A, B, D, E, F and G remain unknown. Many workers on the Bti receptor protein already use AeBBMVWs extensively for experiments but prepare BBMVs from dissected midgut for publications. Finally, AeBBMVWs would enable new studies on Baculovirus receptors (Becnel et al., 2001).

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